

Applicants file an information disclosure statement concurrently herewith.

IN THE SPECIFICATION:

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Please add the following section heading and paragraph directly after the title.

-- CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of United States Provisional Application No. 60/192,176, filed March 27, 2000; United States Provisional Application no. 60/192,179, filed March 27, 2000; United States Provisional Application No. 60/208,538, filed June 1, 2000; and United States Provisional Application No. 60/244,989, filed October 30, 2000.--

Please replace the four (4) consecutive paragraphs beginning on page 14, line 22 and ending on page 15, line 22, with the following four (4) consecutive replacement paragraphs:

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-- Figure 1. *Flow diagram for the generation of modified single-stranded oligonucleotides.* The upper strands of chimeric oligonucleotides I and II are separated into pathways resulting in the generation of single-stranded oligonucleotides that contain (A) 2'-O-methyl RNA nucleotides or (B) phosphorothioate linkages. Fold changes in repair activity for correction of kan^s in the HUH7 cell-free extract are presented in parenthesis. HUH7 cells are described in Nakabayashi et al., Cancer Research 42: 3858-3863 (1982). Each single-stranded oligonucleotide is 25 bases in length and

contains a G residue mismatched to the complementary sequence of the kan^s gene. The numbers 3, 6, 8, 10, 12 and 12.5 respectively indicate how many phosphorothioate linkages (S) or 2'-O-methyl RNA nucleotides (R) are at each end of the molecule. Hence oligo 12S/25G contains an all phosphorothioate backbone, displayed as a dotted line. Smooth lines indicate DNA residues, wavy lines indicate 2'-O-methyl RNA residues and the carat indicates the mismatched base site (G).

Figure 1(C) provides a schematic plasmid indicating the sequence of the kan chimeric double-stranded hairpin oligonucleotide (left; SEQ ID NO: 4341) and the sequence the tet chimeric double-stranded hairpin oligonucleotide used in other experiments (SEQ ID NO: 4342). Figure 1(D) provides a flow chart of a kan experiment in which a chimeric double-stranded hairpin oligonucleotide (SEQ ID NO: 4341) is used. In Figure 1(D), the Kan mutant sequence corresponds to SEQ ID NO: 4343 and SEQ ID NO: 4344; the Kan converted sequence corresponds to SEQ ID NO: 4345 and SEQ ID NO: 4346; the mutant sequence in the sequence trace corresponds to SEQ ID NO: 4347 and the converted sequences in the sequence trace correspond to SEQ ID NO: 4348.

Figure 2. *Genetic readout system for correction of a point mutation in plasmid pK^sm4021.* A mutant kanamycin gene harbored in plasmid pK^sm4021 is the target for correction by oligonucleotides. The mutant G is converted to a C by the action of the oligo. Corrected plasmids confer resistance to kanamycin in *E. coli* (DH10B) after electroporation leading to the genetic readout and colony counts. The wild type sequence corresponds to SEQ ID NO: 4349.

Figure 3: *Target plasmid and sequence correction of a frameshift mutation by chimeric and single-stranded oligonucleotides.* (A) Plasmid pT^sΔ208 contains a single base deletion mutation at position 208 rendering it unable to confer tet resistance. The target sequence presented below indicates the insertion of a T directed by the oligonucleotides to re-establish the resistant phenotype. (B) DNA sequence confirming base insertion directed by Tet 3S/25G; the yellow highlight indicates the position of frameshift repair. The wild type sequence corresponds to SEQ ID NO: 4350, the mutant sequence corresponds to SEQ ID NO: 4351 and the converted sequence corresponds to SEQ ID NO: 4352. The control sequence in the sequence trace corresponds to SEQ ID NO: 4353 and the 3S/25A sequence in the sequence trace corresponds to SEQ ID NO: 4354.

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Figure 4. *DNA sequences of representative kan^r colonies.* Confirmation of sequence alteration directed by the indicated molecule is presented along with a table outlining codon distribution. Note that 10S/25G and 12S/25G elicit both mixed and unfaithful gene repair. The number of clones sequenced is listed in parentheses next to the designation for the single-stranded oligonucleotide. A plus (+) symbol indicates the codon identified while a figure after the (+) symbol indicates the number of colonies with a particular sequence. TAC/TAG indicates a mixed peak. Representative DNA sequences are presented below the table with yellow highlighting altered residues. The sequences in the sequence traces have been assigned numbers as follows: 3S/25G, 6S/25G and 8S/25G correspond to SEQ ID NO: 4355, 10S/25G corresponds to SEQ ID NO: 4356, 25S/25G on the lower left corresponds to SEQ ID NO: 4357 and 25S/25G on the lower right corresponds to SEQ ID NO: 4358.- -

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Please replace the three (3) consecutive paragraphs beginning on page 16, line 4 and ending on page 16, line 17, with the following three (3) consecutive replacement paragraphs:

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Figure 7. *Hygromycin-eGFP target plasmids.* (A) Plasmid pAURHYG(ins)GFP contains a single base insertion mutation between nucleotides 136 and 137, at codon 46, of the Hygromycin B coding sequence (cds) which is transcribed from the constitutive ADH1 promoter. The target sequence presented below indicates the deletion of an A and the substitution of a C for a T directed by the oligonucleotides to re-establish the resistant phenotype. In Figure 7A, the sequence of the normal allele corresponds to SEQ ID NO: 4359, the sequence of the target/existing mutation corresponds to SEQ ID NO: 4360 and the sequence of the desired alteration corresponds to SEQ ID NO: 4361. (B) Plasmid pAURHYG(rep)GFP contains a base substitution mutation introducing a G at nucleotide 137, at codon 46, of the Hygromycin B coding sequence (cds). The target sequence presented below the diagram indicates the amino acid conservative replacement

of G with C, restoring gene function. In Figure 7B, the sequence of the normal allele corresponds to SEQ ID NO: 4359, the sequence of the target/existing mutation corresponds to SEQ ID NO: 4362 and the sequence of the desired alteration corresponds to SEQ ID NO: 4361.

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Figure 8. *Oligonucleotides for correction of hygromycin resistance gene*. The sequence of the oligonucleotides used in experiments to assay correction of a hygromycin resistance gene are shown. DNA residues are shown in capital letters, RNA residues are shown in lowercase and nucleotides with a phosphorothioate backbone are capitalized and underlined. In Figure 8, the sequence of HygE3T/25 corresponds to SEQ ID NO: 4363, the sequence of HygE3T/74 corresponds to SEQ ID NO: 4364, the sequence of HygE3T/74a corresponds to SEQ ID NO: 4365, the sequence of HygGG/Rev corresponds to SEQ ID NO: 4366 and the sequence of Kan70T corresponds to SEQ ID NO: 4367.

Figure 9. *pAURNeo(-)FIAsH plasmid*. This figure describes the plasmid structure, target sequence, oligonucleotides, and the basis for detection of the gene alteration event by fluorescence. In Figure 9, the sequence of the Neo/kan target mutant corresponds to SEQ ID NO: 4343 and SEQ ID NO: 4344, the converted sequence corresponds to SEQ ID NO: 4345 and SEQ ID NO: 4346 and the FIAsH peptide sequence corresponds to SEQ ID NO: 4368.--

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Please replace the paragraph beginning on page 17, line 13 and ending on page 18, line 2 with the following replacement paragraph:

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-- We also construct three mammalian expression vectors, pHyg(rep)eGFP, pHyg(Δ)eGFP, pHyg(ins)eGFP, that contain a substitution mutation at nucleotide 137 of the hygromycin-B coding sequence. (rep) indicates a T137→G replacement, (Δ) represents a deletion of the G137 and (ins) represents an A insertion between nucleotides 136 and 137. All point mutations create a nonsense termination codon at residue 46. We use pHygEGFP plasmid

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(Invitrogen, CA) DNA as a template to introduce the mutations into the hygromycin-eGFP fusion gene by a two step site-directed mutagenesis PCR protocol. First, we generate overlapping 5' and a 3' amplicons surrounding the mutation site by PCR for each of the point mutation sites. A 215 bp 5' amplicon for the (rep), (Δ) or (ins) was generated by polymerization from oligonucleotide primer HygEGFPf (5'-AATACGACTCACTATAGG-3'; SEQ ID NO: 4369) to primer Hygrepr (5'-GACCTATCCACGCCCTCC-3'; SEQ ID NO: 4370), Hyg Δ r (5'-GACTATCCACGCCCTCC-3'; SEQ ID NO: 4371), or Hyginsr (5'-GACATTATCCACGCCCTCC-3'; SEQ ID NO: 4372), respectively. We generate a 300bp 3' amplicon for the (rep), (Δ) or (ins) by polymerization from oligonucleotide primers Hygrepf (5'-CTGGGATAGGTCCTGCGG-3'; SEQ ID NO: 4373), Hyg Δ f (5'-CGTGGATAGTCCTGCGG-3'; SEQ ID NO: 4374), Hyginsf (5'-CGTGGATAATGTCCTGCGG-3'; SEQ ID NO: 4375), respectively to primer HygEGFP_r (5'-AAATCAGCCATGTAGTG-3'; SEQ ID NO: 4376). We mix 20 ng of each of the resultant 5' and 3' overlapping amplicon mutation sets and use the mixture as a template to amplify a 523 bp fragment of the Hygromycin gene spanning the KpnI and RsrII restriction endonuclease sites. We use the Expand PCR system (Roche) to generate all amplicons with 25 cycles of denaturing at 94°C for 10 seconds, annealing at 55°C for 20 seconds and elongation at 68°C for 1 minute. We digest 10 μ g of vector pHyGEGFP and 5 μ g of the resulting fragments for each mutation with KpnI and RsrII (NEB) and gel purify the fragment for enzymatic ligation. We ligate each mutated insert into pHyGEGFP vector at 3:1 molar ratio using T4 DNA ligase (Roche). We screen clones by restriction digest, confirm the mutation by Sanger dideoxy chain termination sequencing and purify the plasmid using a Qiagen maxiprep kit.-

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Please replace the paragraph beginning on page 21,
line 15 and ending on page 22, line 3 with the following
replacement paragraph:

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-- *Correction of a mutant kanamycin gene in cultured mammalian cells.* The experiments are performed using different mammalian cells, including, for example, 293 cells (transformed human primary kidney cells), HeLa cells (human cervical carcinoma), and H1299 (human epithelial carcinoma, non-small cell lung cancer). HeLa cells are grown at 37°C and 5% CO₂ in a humidified incubator to a density of 2×10^5 cells/ml in an 8 chamber slide (Lab-Tek). After replacing the regular DMEM with Optimem, the cells are co-transfected with 10 µg of plasmid pAURNeo(-)FIAsH and 5 µg of modified single-stranded oligonucleotide (3S/25G) that is previously complexed with 10 µg lipofectamine, according to the manufacturer's directions (Life Technologies). The cells are treated with the liposome-DNA-oligo mix for 6 hrs at 37°C. Treated cells are washed with PBS and fresh DMEM is added. After a 16-18 hr recovery period, the culture is assayed for gene repair. The same oligonucleotide used in the cell-free extract experiments is used to target transfected plasmid bearing the kan^s gene. Correction of the point mutation in this gene eliminates a stop codon and restores full expression. This expression can be detected by adding a small non-fluorescent ligand that bound to a C-C-R-E-C-C (SEQ ID NO: 4385) sequence in the genetically modified carboxy terminus of the kan protein, to produce a highly fluorescent complex (FIAsH system, Aurora Biosciences Corporation). Following a 60 min incubation at room temperature with the ligand (FIAsH-EDT2), cells expressing full length kan product acquire an intense green fluorescence detectable by fluorescence microscopy using a fluorescein filter set. Similar

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experiments are performed using the HygeGFP target as described in Example 2 with a variety of mammalian cells, including, for example, COS-1 and COS-7 cells (African green monkey), and CHO-K1 cells (Chinese hamster ovary). The experiments are also performed with PG12 cells (rat pheochromocytoma) and ES cells (human embryonic stem cells).- -

Please replace the two (2) consecutive paragraphs beginning on page 24, line 6 and ending on page 24, line 27 with the following two (2) consecutive replacement paragraphs:

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-- *Oligonucleotides can target multiple nucleotide alterations within the same template.* The ability of individual single-stranded oligonucleotides to correct multiple mutations in a single target template is tested using the plasmid pK^sm4021 and the following single-stranded oligonucleotides modified with 3 phosphorothioate linkages at each end (indicated as underlined nucleotides): Oligo1 is a 25-mer with the sequence TTCGATAAGCCTATGCTGACCCGTG (SEQ ID NO: 4377) corrects the original mutation present in the kanamycin resistance gene of pK^sm4021 as well as directing another alteration 2 basepairs away in the target sequence (both indicated in boldface); Oligo2 is a 70-mer with the 5'-end sequence TTCGGCTACGACTGGGCACAACAGACAATTGGC (SEQ ID NO: 4378) with the remaining nucleotides being completely complementary to the kanamycin resistance gene and also ending in 3 phosphorothioate linkages at the 3' end. Oligo2 directs correction of the mutation in pK^sm4021 as well as directing another alteration 21 basepairs away in the target sequence (both indicated in boldface).

We also use additional oligonucleotides to assay the ability of individual oligonucleotides to correct multiple mutations in the pK^sM4021 plasmid. These include, for example, a second 25-mer that alters two nucleotides that are three nucleotides apart with the sequence 5'-TTGTGCCCAGTCGTAICCGAATAGC-3' (SEQ ID NO: 4379); a 70-mer that alters two nucleotides that are 21 nucleotides apart with the sequence 5'-CATCAGAGCAGCCAATTGTCTGTTGTGCC

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CAGTC**G**TAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGA-3' (SEQ ID NO: 4380); and another 70-mer that alters two nucleotides that are 21 nucleotides apart with the sequence 5'-GCTGACAGCCGGAACACGGCGGCATCAGAGCAGCC**A**ATTGTCTGTTGTGCCCAGTC**G**TAGCCGAATAGCCT-3' (SEQ ID NO: 4381). The nucleotides in the oligonucleotides that direct alteration of the target sequence are underlined and in boldface. These oligonucleotides are modified in the same way as the other oligonucleotides of the invention.- -

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Please replace the paragraph beginning on page 34, line 10 and ending on page 34, line 20, with the following replacement paragraph:

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- -We also use additional oligonucleotides to assay the ability of individual oligonucleotides to correct multiple mutations in the pAURHYG(x)eGFP plasmid. These include, for example, one that alters two basepairs that are 3 nucleotides apart is a 74-mer with the sequence 5'-CTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGG**G**TAC**G**TCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTAC-3' (SEQ ID NO: 4382); a 74-mer that alters two basepairs that are 15 nucleotides apart with the sequence 5'-CTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATAC**G**TCCTGCGGGTAAAC**A**AGCTGCGCCGATGGTTTCTAC-3' (SEQ ID NO: 4383); and a 74-mer that alters two basepairs that are 27 nucleotides apart with the sequence 5'-CTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATAC**G**TCCTGCGGGTAAATAGCTGCGCCGAC**G**GGTTTCTAC (SEQ ID NO: 4384). The nucleotides in these oligonucleotides that direct alteration of the target sequence are underlined and in boldface. These oligonucleotides are modified in the same ways as the other oligonucleotides of the invention.- -
